

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 3

Amendments to the Specification:

Please replace the paragraph beginning at page 4, line 17, with the following rewritten paragraph:

FI
The invention further provides assays, e.g., screening tests, for identifying an agent which modulates the level of one or more myofilament protein modification products in a biological sample, at least one of the myofilament protein modification products being a chemical adduct of a myofilament protein. The assay involves obtaining a biological sample containing a myofilament protein modification product from a subject, testing the biological sample with an agent (e.g., contacting the sample with the agent), and determining the effect of the agent on the level of the myofilament protein modification product in the biological sample, wherein an agent(s) which modulates the level of the myofilament protein modification product in a biological sample ~~are~~ is identified.

~
Please replace the paragraph beginning at page 5, line 18, with the following rewritten paragraph:

FI
Figure 1 shows the results of an SDS-PAGE analysis of reperfusion effluent from rat hearts which had undergone 15 min equilibration followed by 60 min of ischemia. Panel A shows 12.5% SDS polyacrylamide gel of the two minute effluent fractions collecteded at 0 and 2 min. Serum albumin and triose phosphate isomerase were identified by amino acid sequencing (Table 2). Panels B to F show western blots of the combined effluent

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 4

fractions (0 to 4 min) probed with anti- α -actinin (panel B), anti-TnT (panel C), anti-tropomyosin (TM) (panel D), anti-TnI peptide P142T (residues 136 to 148) (MAb E2, panel E) and anti-MLC1 (panel F) antibodies. The MLC1 modification product is indicated by an arrow.

[Please replace the paragraph beginning at page 5, line 26, with the following rewritten paragraph:]

Figure 2 shows the results of an SDS-PAGE analysis of isolated myofibrils from control and globally ischemic rat hearts. Left ventricular tissue samples obtained from isolated rat hearts were placed in saline in plastic bags for 60 min at either 4 °C (control, 1) or 39 °C (global ischemia, 2). Panel A shows the coomassie blue stain of the 12.5% crosslinked gel. Panels B to F show corresponding western blots using anti- α -actinin (panel B), anti-TnI peptide residues 136 to 148 (panel C), anti-TnT (panel D), and anti-MLC1 (panel E) antibodies. Modification products are indicated by arrows. The data reveal a loss of α -actinin in the global ischemic myofibrils.

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 5

Please replace the paragraph beginning at page 6, line 4, with the following rewritten paragraph:

f3
Figure 3 shows an SDS-PAGE analysis of skinned left ventricle tissue samples from isolated rat hearts after 15 min equilibration followed by either 45 min perfusion (control, 1), 15 min ischemia followed by 45 min reperfusion (i.e., 15/45; 2), 60 min ischemia (3) or 60 min ischemia followed by 45 minutes reperfusion (i.e., 60/45; 4). Panel A shows the coomassie blue stain of the 12.5% crosslinked gel. Panels B-F show corresponding western blots using anti- α -actinin (panel B), anti-TnI peptide residues 136 to 148 (MAb E2, panel C), anti-TnT (panel D), anti-TM (panel E), and anti-MLC1 (panel F) antibodies. Panel G shows the western blot of a 10% SDS-PAGE of control tissue and tissue obtained from rats which experienced 60 min ischemia (2). The western blot was probed with anti- α -actinin antibody. Modification products are indicated by arrows.

Please replace the paragraph beginning at page 9, line 1, with the following rewritten paragraph:

f4
Figures 14A and B are gels showing that two distinct dephosphorylated forms of protein-protein complexes are ~~presnce~~ present in human heart.

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 6

[Please replace the paragraph beginning at page 9, line 15, with the following rewritten paragraph:]

To date, the consensus is that there is little free intact cTnI (Giuliani et al. 1999, *Clin. Chem.* **45**:213-222; Wu et al. 1998, *Clin. Chem.* **44**:1198-1208) present in blood, but rather the predominate ~~from~~ form is a cTnI-cTnC complex (Giuliani et al. 1999, *Clin. Chem.* **45**:213-222; Morjana et al. 1998, *Biotechnol. Appl. Biochem.* **28**:105-111; Wu et al. 1998, *Clin. Chem.* **44**:1198-1208), and some or all of the cTnI is proteolyzed from the C-terminus and/or the N-terminus. The modification progression of cTnI and its correlation with increasing severity of ischemia in rat the has been determined: cTnI is initially proteolyzed from the C-terminus (Van Eyk et al. 1998, *Circ. Res.* **82**:261-271; McDonough et al. 1999, *Circ. Res.* **84**:9-20), with loss of 17 amino acids with formation of protein-protein complexes between cTnI degradation products and cTnT or cTnC; this is followed by progressive N-terminal truncation (McDonough et al. 1999, *Circ. Res.* **84**:9-20). Furthermore, it is these modification products, and not intact cTnI that are preferentially detected in the effluent from the severely ischemic, necrosis rat heart. In human myocardium, degradation and protein-protein complex formation is more extensive and complex, due in part to variation in stage and type of disease of patients.

[Please replace the paragraph beginning at page 9, line 29, with the following paragraph:]

The presence of cTnT in serum is also considered a biochemical marker of myocardial damage (Ravkilde 1998, *Dan. Med.*

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 7

Bull. **45**:34-50; Solymoss et al. 1997, *Clin. Cardiol.* **20**:934-42) and comparisons with cTnI and CKMB suggest they are nearly equivalent (e.g., Mair et al. 1995, *Eur. J. Clin. Chem. Clin. Biochem.* **33**:869-72; Stromme et al. 1998, *Scand. J. Clin. Lab. Invest.* **58**:693-699) or that with CKMB and cTnI diagnosis of acute myocardial infarction is more rapid and has higher specificity (Penttila et al. 1997, *Eur. J. Clin. Chem. Clin. Biochem.* **35**:767-74). In skeletal muscle injury, TnI, TnT and their various skeletal isoforms as well as tropomyosin and MLC are ~~proteolyzed~~ proteolyzed and undergo covalent complex formation. Skeletal TnI and other myofilament proteins and various ~~modification~~ modification products are detected in serum obtained from rats with respiratory fatigue/failure (due to breathing against a load). Thus there appears to be a similar or overlapping pathway for muscle damage in both cardiac and skeletal muscle.

FS
Amend

Please replace the paragraph beginning at page 10, line 10, with the following rewritten paragraph:

The detection of myofilament protein modification products, in which those products are intact myofilament proteins, degradation products of myofilament proteins, and protein-protein complexes of myofilament proteins, in various biological samples such as blood, tissue, and urine, was described in detail in our co-pending U.S. Patent Application No. 09/115,589, filed July 15, 1998. This prior application is incorporated herein by reference in its entirety. The present invention represents a substantial improvement in the analysis of myofilament proteins in biological

FS

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 8

samples in that it provides for the detection of chemical adducts of myofilament proteins (e.g., post-translational modifications) and various modifications thereof, including protein-protein complexes and protein fragments thereof. Heretofore, the detection of such chemical adducts of myofilament proteins in blood was not possible, and, although phosphorylated TnI was known to exist in muscle tissue, its significance with respect to muscle damage and heart disease was unknown. In accordance with the invention, ~~progressive~~ progressive post-translational modification (e.g., phosphorylation) of myofilament proteins, their degradation and covalent complexes can be monitored, and correlated with extent of injury and type and stage of muscle damage (e.g., heart disease) of patients.

[Please replace the paragraph beginning at page 10, line 25, with the following rewritten paragraph:]

fs We have developed a method for detecting myofilament proteins such as troponins (TnI, TnT, TnC) and their modification products by western blot analysis of human serum directly (see Examples II and III, below). This method overcomes problems due to: 1) the large quantity of IgG and albumin which overwhelm the extremely small quantity of troponin; 2) manipulation of the serum used to either concentrate or isolate these proteins; and 3) bias and sensitivity limitations due to ~~immunogenicity~~ immunogenicity or detection methods. Using this method we have shown that, for example, TnI in the serum of patients following bypass surgery can be detected even below the level of TnI detected using currently available diagnostic kits. In one embodiment of this invention (see Example II, below), this method

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 9

FS
cont
of western blot analysis was used to detect modified forms of TnI in serum from MI patients for up to 5 days following admittance to the emergency room, and to characterize changing patterns of TnI modification with the time course of MI.

Please replace the paragraph beginning at page 12, line 8, with the following rewritten paragraph:

fb
According to this embodiment, a change with time in the presence or amount of one or more myofilament protein modification products is indicative of the extent or severity of the disease state of the subject. Also in accordance with this embodiment, such a change with time can be used to monitor the condition of a subject being treated for heart disease, and for evaluating the efficacy of treatments for such conditions. It will be appreciated that the number of biological samples obtained from a subject, and the time elapsed between samples, will vary in accordance with the condition of the subject and the diagnostic situation. For example, prior to and during heart surgery it is preferred to take multiple samples frequently, such as from 30 min to 2 h apart, to monitor the subject's response to the treatment. During recovery, biological samples can be taken less frequently, such as every 2 to 24 h. After recovery, samples can continue to be obtained even less frequently, such as once per month, to provide feedback as to the subject's condition, and thus to provide an indication of the efficacy of the surgery, or whether the subject is developing further complications, such as heart failure. In other situations, for example where a subject shows a ~~prediposition~~ predisposition for MI or HF, biological samples can be regularly obtained such as

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 10

weekly, biweekly, or monthly, to determine the subject's prognosis on an on-going basis, and to evaluate the efficacy of any treatment(s) being administered to the subject. Furthermore, in the case of cardiac transplantation, patients can be monitored prior to surgery to ~~determine~~ determine the severity of their condition, and during surgery to detect the onset of rejection of the transplanted heart as well as the effectiveness of anti-rejection ~~therapy~~ therapy. With heart ~~transplantation~~ transplantation a patient typically undergoes 10 to 20 biopsies in the first year. Monitoring changes in different post-translational myofilament protein modification products in the serum in accordance with the present invention could ~~elimant~~ eliminate the need for biopsies. For respiratory (skeletal muscle) injury, identification of patients experiencing difficulty being weaned from respirators, or the degree of respiratory muscle damage with disease such as COPD or asthma, could be carried out in accordance with the invention either as single or multiple sequential assays. With sepsis, where there is ~~multiple~~ multiple organ damage, or even heart failure, and where respiratory (skeletal) muscle injury can also occur, the invention provides for monitoring and differentiating both skeletal and cardiac muscle damage via assessing the level and quantity of post-translational modification of specific myofilament proteins, to assess severity of disease.

Please replace the paragraph beginning at page 14, line 11, with the following rewritten paragraph:

The phrase "chemical adducts" or "chemical adducts of myofilament protein(s)" is defined as a peptide species formed by

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 11

* new matter?
bonding, for example covalent bonding, of a polypeptide or a polypeptide fragment and a *different* compound. For purposes of this disclosure, chemical adducts do not include covalent linkage of two similar species, i.e., protein-protein complexes. However, a chemical adduct which is the linkage of a different chemical compound or moiety to a protein-protein complex or a protein degradation product is encompassed by this definition of chemical adduct. Chemical adducts known in the art relating to post-translational modification of proteins include, but are not limited to, phosphorylation, glycosylation, myristylation, phenylation, acetylation, nitrosylation, s-glutathiolation, amidation, biotinylation, c-mannosylation, flavinylation, farnesylation, formylation, geranyl-geranylation, hydroxylation, lipoylation, methylation, palmitoylation, sulphation, gamma-carboxyglutamic acids, N-acyl diglyceride (tripalmitate), O-GlcNAc, pyridoxal phosphate, phospho-pantetheine, and pyrrolidone carboxylic acid. Preferred chemical adducts are phosphorylation, glycosylation, myristylation, phenylation, acetylation, nitrosylation, and sulphation. Chemical adducts of myofilament proteins include such post-translational modification of intact myofilament proteins, of degradation products of myofilament proteins, and of protein-protein complexes of myofilament proteins.

P7 cont

Please replace the paragraph beginning at page 15, line 19, with the following rewritten paragraph:

P8
The term "muscle damage" is defined as cellular damage in skeletal muscle and in the myocardium as a result of hypoxia, hypoxemia, ischemia and/or ischemia/reperfusion injuries, as well

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 12

as any insult or stress that activates or is associated with activation of a protease and/or a cross-linking enzyme such that modification (e.g., cross-linking, degradation) of cardiac myofilament proteins occurs. Muscle damage may be acute, where it can result from any brief (acute) ischemic/hypoxic period (e.g., 30 seconds to 2 days) such as stunning, or pre-conditioning such as infarction (e.g., myocardial infarction (MI)), unstable angina and the like. In some cases, such as in stunning, acute muscle damage may be reversible. Muscle damage may also be chronic, where it can result from longer (chronic) ischemic/hypoxic episodes (e.g., durations of days to years); such as heart failure (HF) and diabetes. Chronic muscle injury includes situations where muscle injury (e.g., due to necrosis or ~~apoptosis~~ apoptosis and loss of muscle cells) causes the muscle to have to ~~compensate~~ compensate for loss of functioning muscle cells. This leads to hypertrophy or atrophy of the muscle. Under these conditions, post-translational ~~modification~~ modification occurs to ~~specific~~ specific myofilament proteins in a time dependent manner.

FB
cont

Please replace the paragraph beginning at page 16, line 21, with the following rewritten paragraph:

Chronic injury to the heart will cause remodelling and hypertrophy, with loss via necrosis or ~~apoptosis~~ apoptosis therefore allowing monitoring by serum samples and when possible tissue samples (at time of heart surgery if appropriate). Low levels of myofilament proteins and their modification products and chemical adducts can be detected in blood and presence or quantity or quality of a myofilament protein(s) or its

pg

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 13

PP
cont

modification product indicate stage of disease. TnI and other myofilament proteins are modified both at gene level (upregulation) and with post-translational modification including phosphorylation and possibly glycosylation at unique sites compared to those with acute injury (right after injury). For example, an increase in amount of TnC in tissue (and therefore the amount present in blood) correlates with a decrease in heart function. In other words, the more TnC present the lower the cardiac output or ejection fraction. TnT does not undergo an isoform switch (in swine ischemic induced HF) but there is a large increase in its pI (from ~4.5 to 6.5) due to a post-translational modification.

Please replace the paragraph beginning at page 18, line 6, with the following rewritten paragraph:

FP

The term "biological sample" is intended to include any sample obtained from a subject which may contain a myofilament protein modification product as defined above detectable by the methods of the present invention. In one embodiment, the biological sample is a sample of a tissue derived from a subject, preferably a sample of a cardiac or skeletal muscle tissue. The sample can be a whole tissue or part of a tissue retaining the myofilament protein modification product. For example, a small biopsy tissue from a subject undergoing heart surgery or a sample obtained via catheterization following heart transplantation can be used in the method of the invention. Alternatively, the biological sample can be a biological fluid such as whole blood, serum, plasma, lymphatic fluid, amniotic fluid, cerebrospinal fluid, urine, and the like. Fluid extracts of tissues such as

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 14

*Pro
cont*

heart or skeletal muscle can also be used in the method of the present invention. The preferred biological fluid for this invention, however, is blood, serum or urine.

Please replace the paragraph beginning at page 19, line 3, with the following rewritten paragraph:

§11

Assessment of myocardial or skeletal muscle damage in a biological sample can be performed by direct detection of myofilament protein modification product(s) in the sample, using, for example, chromatography techniques such as HPLC, electrophoresis, ELISA, RIA analysis (immunological detection), or peptides or proteins that bind to myofilament proteins. These analyses are used to detect differences between elution profiles of samples obtained before and after, for example, treatment of hypoxemia, hypoxia, ischemia or ischemia/reperfusion. As well, the appearance or disappearance of one or more myofilament protein modification products, such as chemical adducts, peptides, or fragments, such as, for example, phosphorylated versus non phosphorylated intact human cardiac TnI or TnI residues 1-192 or 193-209 (or any C-terminal and/or N-terminal fragment), or myosin light chain ⁽¹⁾ residues 1 to 19, in the elution profiles obtained during HPLC analysis can be used as indicators of muscle damage.

*new
matter?*

[Please replace the paragraph beginning at page 19, line 15, with the following rewritten paragraph:]

It will be appreciated that, for purposes of detecting specific myofilament protein modification products in accordance with the invention, using standard techniques, phospho-specific

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 15

F11
cont

antibodies can be produced that recognize phosphorylated or non-phosphorylated versions of, for example, TnI, TnT, and tropomyosin and their degradation products. Further, specific antibodies can be produced that only recognize specific phosphorylated amino acid residues. The latter is important for determining whether a particular residue or novel site is involved, and for assessing the type and state of a disease.

Please replace the paragraph beginning at page 28, line 6, with the following rewritten paragraph:

F12

For example, when qualitatively characterizing different myofilament proteins and/or modification products present in the biological sample, antibodies can be used which differentially recognize epitopes present in the various modification products. Using a label that has a measurable moiety attached to it (e.g., b-galactosidase), a profile or "fingerprint" of the proteins and modification products can be obtained. This profile, which is expected to include, for example, characteristic ratios of various proteins and/or fragments from the same (e.g., cardiac TnI residues 1 to 193 vs. cardiac TnI residues 63 to 193) or from different (e.g., TnI vs. myosin light chain I) proteins, can then be associated with a level (i.e., extent) or type of myocardial damage. In ~~addition~~ addition, the quantity of phosphorylated vs dephosphorylated forms of TnI or other myofilament ~~protiens~~ proteins can indicate severity of disease, time from initial insult, or the extent of damage that has occurred. For example, the appearance of covalent complexes or small TnI fragments may indicate chronic long term damage to the heart. Identifying

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 16

PH2
cont
phosphorylation of many residues or specific amino ~~acid~~ acid residues also helps in stratifying the severity or type of disease. Chemical ~~modifications~~ modifications other than phosphorylation occur to ~~myofialemt~~ myofilament proteins, in particular to TnT with certain disease states.

Please replace the paragraph beginning at page 29, line 1, with the following rewritten paragraph:

PH3
In one embodiment, the method of the invention is used to diagnose mild ischemia by detecting the presence of phosphorylated skeletal or cardiac troponin I or TnI fragments (e.g., cardiac TnI residues 1 to 193) and comparing the levels of this fragment to the levels of intact troponin I and/or comparing levels of ~~phosphroylated~~ phosphorylated to dephosphorylated forms. The quantity of each modification product and chemical adduct, or of the exact amino acid residues that have been ~~modified~~ modified can also be determined. For example, in isolated rat hearts with mild ischemia (stunning), a TnI 22 kDa degradation product is ~~phosphoryalted~~ phosphorylated at a site that is not ~~phsophroylated~~ phosphorylated in the intact protein. As well, phosphorylated TnI and/or its proteolytic fragments may appear in blood or form prior to the unphosphorylated TnI or its proteolytic fragments or ~~protein~~ protein-protein complex.

[Please replace the paragraph beginning at page 29, line 11, with the following rewritten paragraph:]

More severe ischemic injury would involve detection of TnI proteolyzed at the N-terminus or specifically phosphorylated at

Attorney Docket No.: PTQ-0028
Inventors: Van Eyk et al.
Serial No.: 09/419,901
Filing Date: October 18, 1999
Page 17

the N-terminus. With chronic conditions, TnI protein-protein complex is heavily ~~phosphorylated~~ phosphorylated and present in larger quantities than immediately following injury. ~~Furthermore~~ Furthermore, TnT and other myofilament proteins undergo both upregulation (increase in quantity) and specific phosphorylation that may not occur during acute injury.

[Please replace the paragraph beginning at page 29, line 23, with the following rewritten paragraph:]

FB cont
~~As different~~ Different myofilament proteins are more or less susceptible to modification depending on the extent of ischemic or ischemia/reperfusion injury that has occurred. Thus, the appearance of a certain modification to a specific protein can be used as a marker/index for extent of muscle damage. For example, TnT, TnI, α -actinin, or MLC1 degradation (residues 20 to_199) occurs only with very severe ischemia in the myocardium. Therefore, if one detects smaller fragments of TnT, TnI, α -actinin or MLC1 in a biological sample, it is an indication that the myocardium is severely and possibly irreversibly damaged.
